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14. ABSTRACT The complexity of breast cancer demands a systems level understanding of aberrant biochemical signaling in order to develop smart therapeutic targets. This study was undertaken to identify the binding partners of the ERM family protein, ezrin, in normal and metastatic breast cancer cells. We report the generation of ezrin constructs that were tagged at the C- and N-terminus to enable tandem affinity purification of ezrin protein complexes. Mutagenesis of ezrin at key phosphorylation sites was performed to create inactive and constitutively active versions of the protein (T567D, T567A, Y353F, Y477F, S66A) that are expected to differ in their ability to bind to cellular proteins. Overexpression of mutant ezrin protein was tested in the highly metastatic breast cancer cell line 4T1. Overexpression of ezrin mutants did not affect the basal activity of MAPK and PI3K in 4T1 cells. We have completed the optimization of tandem affinity purification protocol for purification of TAP-tagged ezrin and identification by mass spectrometry. In future, we aim to generate 4T1 transfectants stably expressing wild type or mutant versions of ezrin, and purify ezrin complexes to identify their components in normal breast cells and the 4T1 breast cancer cells. The identification of ezrin-interacting proteins will allow elucidation of aberrant signaling pathways in breast cancer cells and offer novel therapeutic targets for the treatment of breast cancer.					
15. SUBJECT TERMS Breast cancer, proteomics, ezrin, signaling					
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FINAL REPORT

Title: A systems biology approach to evaluate ezrin as a therapeutic target in breast cancer.

PI: Neetu Gupta, Ph.D.

Introduction

The complexity of breast cancer demands a systems level understanding of aberrant biochemical signaling in order to develop smart therapeutic targets. Several studies have reported a correlation between the expression of ezrin and development of many types of human malignancies and metastasis. In particular, a strong correlation exists between ezrin expression and histological grade and clinical outcome in breast cancers. Existing breast cancer therapies target proteins whose effects are limited to certain subsets of breast cancer patients. We hypothesize that the association of ezrin with different cellular proteins governs different functional outcomes in breast cancer cells as compared to normal breast epithelial cells. We further suggest that ezrin uses phosphorylated residues to interact with particular signaling players in normal breast epithelial cells and that these interactions are abnormal in breast cancer cells, contributing to tumor growth, survival and metastasis. Our aim is to identify ezrin-specific protein interactions in normal and cancerous breast cells using multiplexed quantitative proteomics. Wild type and mutant forms of ezrin will be tagged for tandem affinity purification (TAP). The TAP-tagged wild type and mutant proteins will be expressed in normal and cancerous breast cells, and ezrin-containing protein complexes will be isolated by TAP. The complexes will be subjected to differential labeling with iTRAQ reagents followed by tandem mass spectrometry for identification and quantification of binding partners. Our research will enable a systems level understanding of the abnormal cell biology of breast cancer cells, and provide insights into the regulation of tumor growth and metastasis by ezrin.

Body

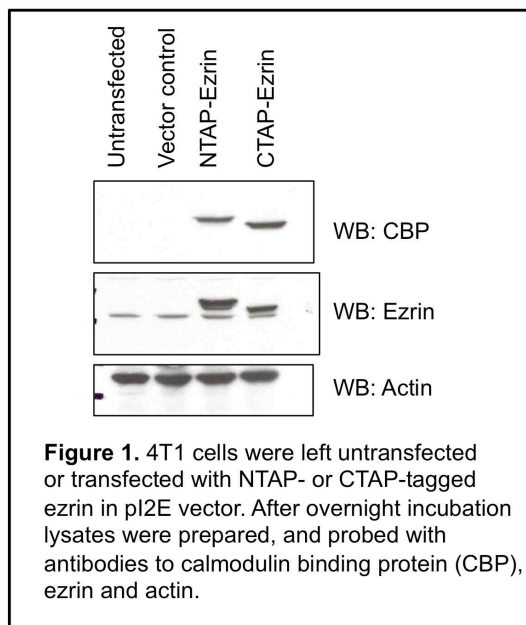
MAJOR RESEARCH TASKS PROPOSED AND ACHIEVED:

(1) Mutagenesis of TAP-tagged wild type ezrin in pIRES2EGFP vector to generate the panel of TAP-tagged mutants (T567D, T567A, S66A, Y353F, Y477F):

Mutagenesis at all the proposed sites is complete. The constructs have been subjected to quality control using restriction digestion and DNA sequencing. Mutations were confirmed by DNA sequencing.

(2) Stable expression of TAP-tagged wild type ezrin and mutants in normal breast epithelial cells and 4T1 cells:

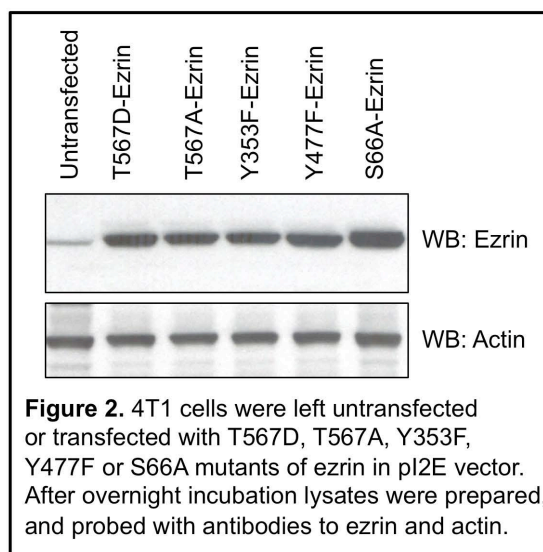
The expression of N- and C-terminal TAP-tagged wild type ezrin was tested in the highly metastatic breast cancer cell line 4T1 cells by nucleofection, followed by flow cytometry and western blotting. The transfection efficiency of the N-TAP and C-TAP constructs was 37% and 41%, respectively. Both proteins showed overexpression, however, a difference in size was observed between the N-TAP and C-TAP ezrin fusion proteins (Fig. 1). This may be due to proteolytic processing of the C-TAP-tagged protein.



Expression of the mutant ezrin plasmids was similarly tested by transient expression in 4T1 cell line. The DNA constructs were transfected into 4T1 cells by nucleofection using vendor recommended conditions. Expression was monitored by flow cytometry for GFP fluorescence and western blotting with ezrin antibody. The transfection efficiency for T567D, T567A, Y353F, Y477F and S66A mutants was 4%, 2.7%, 3%, 5.2% and 8%, respectively. Despite the low transfection efficiency, western blotting of lysates obtained from 4T1 transfectants with ezrin antibody showed 3-4 fold overexpression of the mutants while the expression of actin was unchanged (Fig. 2).

The effect of the mutant proteins on basal levels of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways was evaluated by comparing the phosphoERK1/2 and phosphoAkt levels in untransfected 4T1 cells and those transfected with the T567D, T567A, Y353F and Y477F mutants. There was no significant change in the activation of Erk1/2 and Akt upon expression of the mutants (Fig. 3). The effect of stimuli such as epidermal growth factor (EGF) on mutant ezrin-expressing 4T1 transfectants remains to be tested.

The transiently transfected 4T1 cells were propagated in G418-containing growth medium to select stably expressing clones. Unfortunately, repeated attempts to obtain stable transfectants during the grant period have proven unsuccessful so far. Transient transfection does not provide sufficient numbers of cells necessary for mass spectrometric analyses. Therefore, it was not possible to attempt isolation of wild type and mutant ezrin protein complexes from 4T1 breast cancer cells and mass spectrometric identification of binding partners. We will continue to attempt generation of stable transfectants of 4T1 cells expressing TAP-tagged ezrin mutants, by varying growth and selection conditions, or by changing the breast cancer cell line. However, in an independently funded project in the lab we have been able to create stable transfectants of a B lymphoma cell line expressing TAP-tagged wild type ezrin. We spent



the remaining three months of the grant period in optimizing conditions for tandem affinity purification (TAP) of ezrin from B cell lines (see sections 3, 4 and 5). In future, when we have generated breast cancer cell lines stably expressing ezrin mutants, we will be able to apply TAP to these cells and proceed with the experiments proposed in the DOD grant.

(3) Tandem affinity purification of ezrin and mutant ezrin complexes from normal and cancer cell transfectants.

Stable transfectants of the Bal17 B cell line expressing TAP-tagged wild type ezrin were generated after nucleofection and selection in G418-containing growth medium. High GFP-expressing cells were isolated using a cell sorter to obtain highest expression of TAP-tagged ezrin (Fig. 4).

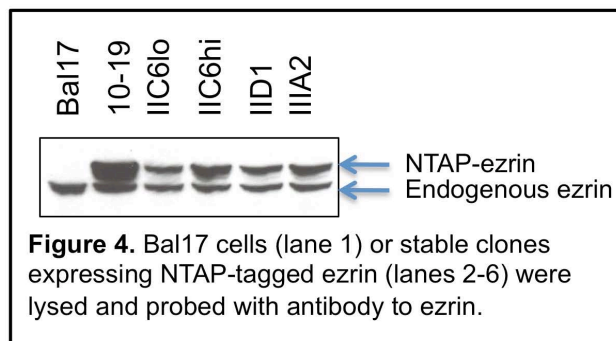
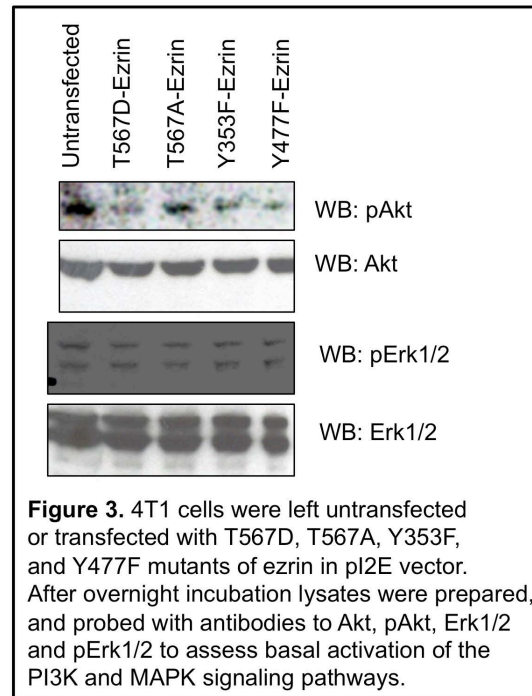
The C-terminal TAP-tagged ezrin (CTAPEz) cell line was used to prepare lysates and subjected to TAP using the manufacturer's (Stratagene) protocol. The purity and complexity of proteins in the purified CTAPEz was analyzed by SDS-PAGE (Fig. 5a) and western blotting (Fig. 5b). The area corresponding to CTAPEz was submitted to the Cleveland Clinic Proteomics Core for mass spectrometric verification of the protein. Briefly, the excised portion was subjected to in-gel digestion with trypsin to generate peptide fragments, followed by liquid chromatography and tandem mass spectrometry. Both, sequences of the tags calmodulin binding peptide (CBP) and streptavidin binding peptide (SBP), and ezrin were identified in the excised band confirming the presence of CTAPEz in the purified preparation.

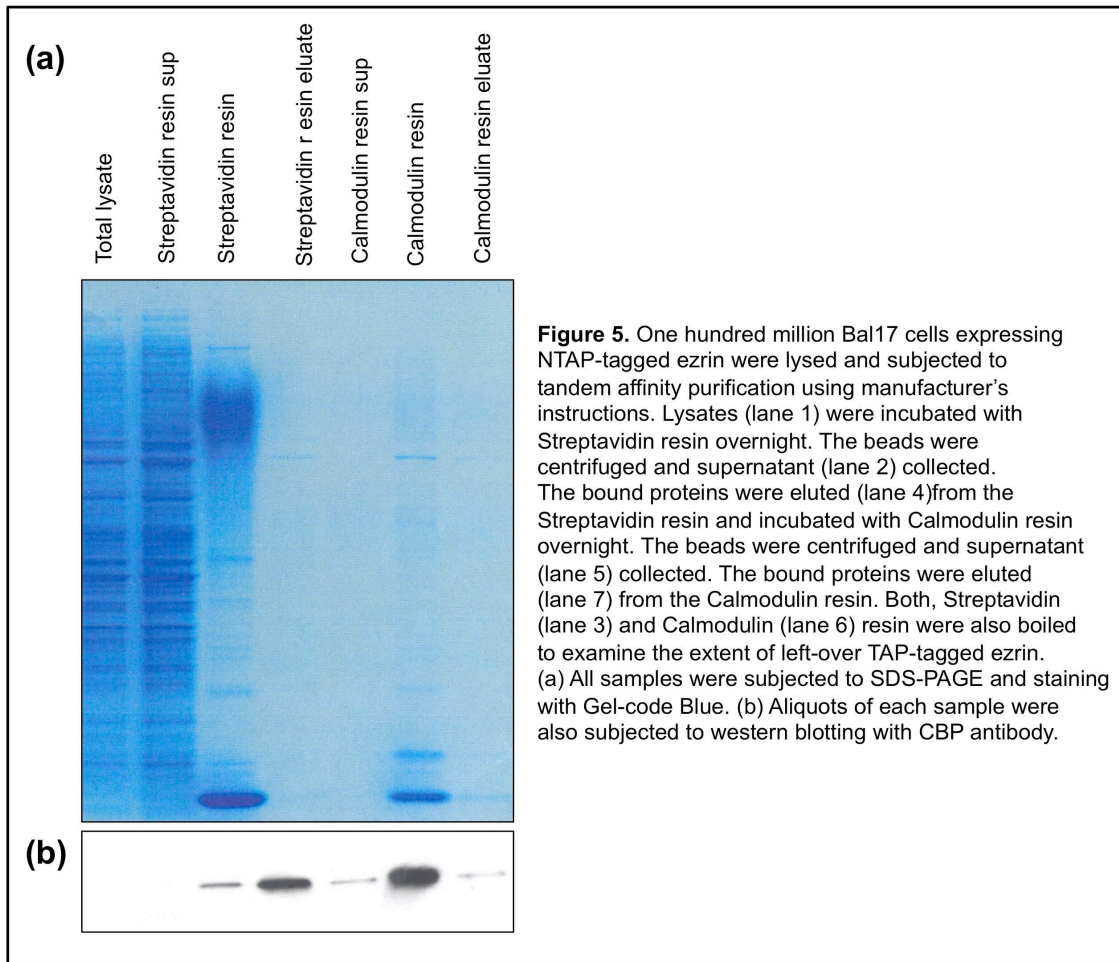
(4) Processing of complexes for iTRAQ labeling and LC-MS/MS analysis.

Could not be undertaken for 4T1 cells due to reasons described above.

(5) Data processing including sequence searches, identification, and quantification.

Could not be undertaken for 4T1 cells due to reasons described above.





Key Research Accomplishments

1. Generation of TAP-tagged ezrin at the C- and N-terminus.
2. Mutagenesis of ezrin at key phosphorylation sites to create inactive and constitutively active protein (T567D, T567A, Y353F, Y477F, S66A)
3. Testing overexpression of mutant ezrin protein in the highly metastatic breast cancer cell line 4T1.
4. Testing the effect of overexpression of ezrin mutants on basal activity of MAPK and PI3K in 4T1 cells.
5. Optimization of tandem affinity purification protocol for purification of TAP-tagged ezrin and identification by mass spectrometry.

Reportable Outcomes

1. Generation of TAP-tagged ezrin constructs
2. Generation of phosphorylation site mutants of ezrin (T567D, T567A, Y353F, Y477F, S66A).

Conclusions

The sites of protein-protein interaction on ezrin likely include the phosphorylation sites at T567, Y353, Y477 and S66. These sites have been reported to undergo phosphorylation in a variety of cells in response to growth factor stimulation. We hypothesize that ezrin interacts with cellular signaling proteins differently in normal and breast cancer cells. In order to identify signaling proteins that interact with ezrin in normal and cancerous epithelial cells, we tagged ezrin at the N- or C-terminus with CBP and SBP and show overexpression of tagged proteins in transient transfection assays. We also generated mutants of ezrin at the phosphorylation sites to enable loss of interaction and gain of interaction studies. The mutants were shown to overexpress in transient expression systems but stably expressing cell lines could not be generated. We plan to try different conditions including different growth and selection media in order to generate stable transfectants so that the remaining part of the proposed study can be carried out, resulting in identification of ezrin interactors in normal and cancerous breast cells.

References

None cited.

Appendices

CURRICULUM VITAE

NEETU GUPTA, PH.D.

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Lerner Research Institute
Cleveland Clinic Foundation
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Cleveland, OH 44195

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EDUCATION

Ph.D, Biochemistry (1990 – 1995), Indian Institute of Science, Bangalore, India

M.Sc, Biotechnology (1988 – 1990), Madurai Kamaraj University, Madurai, India

B.Sc (Honors), Zoology, (1985 – 1988), Delhi University, New Delhi, India

PROFESSIONAL APPOINTMENTS/RESEARCH EXPERIENCE

- Case Comprehensive Cancer Center, Cleveland, OH** 2009
 –
Associate Member
- Cleveland Clinic Foundation, Cleveland, OH** 2007
 –
 Cleveland Clinic Lerner College of Medicine
Assistant Professor
Role of membrane-cytoskeletal dynamics in the regulation of B cell function.
- Cleveland Clinic Foundation, Cleveland, OH** 2007
 –
 Department of Immunology
Assistant Staff
Role of membrane-cytoskeletal dynamics in the regulation of B cell function.
- University of California, San Francisco, San Francisco, CA** 2004
 – 2007
 Laboratory of Dr. Anthony DeFranco
Assistant Researcher
Spatial organization of antigen receptor-mediated signal transduction in B lymphocytes.
- University of California, San Francisco, San Francisco, CA** 1999
 – 2004
 Laboratory of Dr. Anthony DeFranco
Postdoctoral fellow
Spatial organization of antigen receptor-mediated signal transduction in B lymphocytes.
- National Institutes of Health, Rockville, MD** 1995
 – 1998
 Laboratory of Dr. Eric Long
Postdoctoral fellow
Signal transduction mediated by inhibitory receptors on immune cells.
- Indian Institute of Science, Bangalore, India** 1990
 – 1995
 Laboratory of Dr. P. V. SubbaRao
Ph.D. candidate
Thesis on "Immunochemical characterization of the major cross-reacting allergens from the pollen of Parthenium hysterophorus".
- Madurai Kamaraj University, Madurai, India** 1989
 – 1990
 Laboratory of Dr. V.R. Muthukaruppan

M.Sc. student

Dissertation on "Purification of the major serum antigen from the patients of Eales' disease".

National Institute of Immunology, New Delhi, India

May –

Aug, 1989

Laboratory of Dr. Pramod Khandekar

Summer trainee

Subcloning of the major hepatitis B viral antigen from patients with hepatocellular carcinoma.

HONORS & AWARDS

Junior Faculty Travel Award to attend the 96th Annual AAI Meeting, Seattle, WA
May, 2009.

Travel award by Invitrogen for poster presentation at the Gordon Research Conference on "Macromolecular Organization and Cell Function", Oxford, UK, July, 2008.

Travel Award to attend the ASCB-ECF Joint Meeting on "Dynamic Interplay Between Cytoskeletal and Membrane Systems", Dijon, France, June, 2007.

Travel Award to attend the Keystone Symposium on "Lipid Rafts and Cell Function", Steamboat Springs, CO, March, 2006.

K01 Mentored Research Career Development Award from NIDDK, NIH (DK068292) – 2004-2009.

Training grant of the G. W. Hooper Foundation, UCSF – 2002-2004.

NIH Fellows Award for Research Excellence – 1997.

National Institutes of Health Fogarty fellowship – 1995-1998.

Best Poster Award at the XX Annual Conference of Indian Immunology Society, December 17-20, 1993 at Bhopal, India.

Scholarship award of the University Grants Commission, India – 1991.

Ayyamperumal Pillay Endowment Gold Medal for first rank in Biotechnology (M.Sc.) – 1990.

Prof. E.R.B. Shanmugasundaram Endowment Gold Medal for first rank in Biological Sciences (M.Sc.) – 1990.

MEMBERSHIP OF PROFESSIONAL SOCIETIES

Member – American Society for Cell Biology (ASCB).
Member – American Association of Immunologists (AAI).
Member – American Association for the Advancement of Science (AAAS).
Member – Association for Women in Science (AWIS), National and San Francisco chapter.
Member – American Society of Hematology (ASH).

TRAINING/TEACHING EXPERIENCE

Cleveland Clinic

◆ Trained fellows, students, and technicians at Cleveland Clinic

Fellows

Ken Matsui – Postdoctoral fellow, Jul 2009 – till date
Sonal Uppal – Postdoctoral fellow, Apr 2009 – till date
Soumya Panigrahi – Postdoctoral fellow, Jan-May 2009
Nayer Bagheri – Postdoctoral fellow, Sep 2008 – 2009
Neetha Parameswaran – Postdoctoral fellow, Sep 2007 – till date

Students

Laura Davison – Rotation student, Molecular Medicine Ph.D. program, Aug-Oct, 2009
Neilay Shah – High school Summer Student, Jun-Jul, 2009
Meridith Ginesi – Undergraduate Summer Student, Jun-Jul, 2009
Angela Shih – Medical student (Cleveland Clinic Lerner College of Medicine), Jul-Sep, 2008
James Harrington – Undergraduate Summer Student, May-Aug, 2008
Kourtney Nickerson – Rotation student, Molecular Medicine Ph.D. program, Feb-Apr, 2008

Technicians

Alex Liggett – Research technician, Sep 2009 – till date
Afshan Hussain – Research technologist, Sep 2007 – Aug 2008

◆ Lectured students in the Molecular Medicine Ph.D. program – Jan 2008, Sep 2008

◆ Service – Chair, Immunology Seminar Series Steering Committee, 2008 – till date

Judge, Poster presentation at the Annual Retreat of the Lerner Research Institute, 2009

University of California, San Francisco

◆ Mentored rotation students working with me on the B cell signaling project, UCSF, San Francisco.

Michael Patnode – Aug-Oct, 2006.

◆ Participated in the UCSF summer research training program and trained undergraduate students working on projects addressing the role of spatial organization in B cell activation.

Bobby Sahachartsiri, University of California, Los Angeles, CA – Jun-Aug, 2006

Brianna Jang, Stanford University – Jul-Sep, 2004

Martin Szmulewicz, Colorado College, CO – Jun-Aug, 2002

Lamorris Loftin, University of North Carolina, Chapel Hill – Jun-Aug, 2000

Christina Chao, Mount Saint Mary's College, Los Angeles – Jun-Aug, 1999

◆ Trained and directly supervised/mentored technicians working on the B cell antigen receptor signaling project, UCSF, San Francisco.

Barbara Scheer – Jul 2003 to Feb 2006.

Shiloh Martin – Dec 2000 to Jun 2003.

◆ Service (Immunology postdoctoral fellows committee) – Coordinated meetings of postdoctoral fellows with visiting faculty members invited to lecture in the Immunology Seminar Series (2004 – 2007).

RESEARCH SUPPORT

Ongoing research support

1. "Role of Lyn in SLE-like autoimmune disease in mice."

Principal Investigator: Neetu Gupta

Agency: NIH, National Institute for Diabetes, Digestive and Kidney Diseases

Type: K01 (DK068292).

Period: August 15, 2004 - June 30, 2009.

Percentage effort: 50%

2. "Regulation of B cell immune response by the ERM family protein Ezrin."

Principal Investigator: Neetu Gupta

Agency: Cancer Research Institute

Type: Investigator Award
Period: July 2008 – June 2012
Percentage effort: 5%

3. “Regulation of B cell function by membrane-cytoskeletal remodeling.”
Principal Investigator: Neetu Gupta
Agency: NIH, National Institute for Allergy and Infectious Diseases
Type: R01 (AI081743).
Period: July 2009 – June 2013
Percentage effort: 25%

Completed research support

1. “A systems biology approach to evaluate ezrin as a therapeutic target in breast cancer.”
Principal Investigator: Neetu Gupta
Agency: Department of Defense, Breast Cancer Research Program
Type: Concept Award
Period: September 2008 – August 2009
Percentage effort: 10%

INVITED SEMINARS

Cleveland State University, Cleveland, OH – “Membrane-cytoskeletal dynamics in B cell activation”, April 2009.

Case Western Reserve University, Cleveland, OH – “Membrane-cytoskeletal dynamics in B cell activation”, February 2008.

National Institute of Immunology, New Delhi, India – “Spatial organization and regulation of B cell activation”, March 2007.

Indian Institute of Science, Bangalore, India – “Spatial organization and regulation of B cell activation”, April 2007.

RESEARCH PRESENTATIONS AT CONFERENCES

Oral

Microscopy & Microanalysis 2009, Richmond, VA, July 2009 (*Invited Platform Speaker*).

Annual Meeting of the American Association of Immunologists, Seattle, WA, May 2009.

Gordon Research Conference on "Macromolecular Organization and Cell Function", Oxford, UK, July 2008.

NIDDK "Young Investigators meeting", Adelphi, MD, May 2006.

Keystone Symposia on "Lipid Rafts and Cell Function", Steamboat Springs, CO, March 2006.

International Congress of Immunology, Montreal, Canada, July 2004.

FASEB Summer Research Conference on "Immunoreceptors", Tucson, AZ, August 2002.

Annual Immunology Retreat of UC San Francisco and UC Berkeley, Lake Tahoe, CA, November 2001.

Keystone Symposia on "B cell Immunobiology and Disease", Snowbird, UT, April 2001.

Keystone Symposia on "Tolerance and Autoimmunity", Keystone, CO, April 1997.

Poster

Annual meeting of the American Society for Cell Biology, San Francisco, CA, December 2008.

Keystone Symposia on "Lymphocyte Activation and Tolerance", Snowbird, UT, January 2008.

FASEB Summer Research Conference on "Signal transduction in the immune system", Snowmass Village, CO, June 2005.

Keystone Symposia on "Molecular Cell Biology of Lipid Domains", Vancouver, Canada, March 2004.

Annual Meeting of the American Association of Immunologists, Denver, CO, May 2003.

Annual meeting of the American Society for Cell Biology, San Francisco, CA, December 2002.

Annual meeting of the American Society for Cell Biology, Washington DC, December 2001.

Annual Immunology Retreat of UC San Francisco and UC Berkeley, Lake Tahoe, CA, November 2001.

Keystone Symposia on "B cell Immunobiology and Disease", Snowbird, UT, April, 2001.

FASEB Summer Research Conference on "Lymphocytes and Antibodies", Saxtons River, VT, June, 2000.

Keystone Symposia on "Signaling 2000", Keystone, CO, January 2000.

Annual Cell Biology Retreat of University of California-San Francisco, Wilbur Springs, CA, May 1999.

Keystone Symposia on "B Lymphocyte Biology and Disease", Taos, NM, February 1999.

Gordon Research Conference on "Second Messengers & Protein Phosphorylation", Meriden, NH, 1998.

Keystone Symposia on "Signal Transduction and Lipid Second Messengers III", Taos, NM, February 1998.

Keystone Symposia on "Tolerance and Autoimmunity", Keystone, CO, April 1997.

BIBLIOGRAPHY

Research publications

Neetha Parameswaran, Twishasri Dasgupta, and **Neetu Gupta**. 2009. Orchestration of B lymphocyte activation and motility by the ERM protein Ezrin. *Proc. Microsc. Microanal.* **15 (Suppl 2)**.

Neetu Gupta, Bernd Wollscheid, Julian Watts, Barbara Scheer, Ruedi Aebersold, and Anthony L. DeFranco. 2006. Quantitative proteomic analysis of B cell lipid rafts reveals a role for ezrin in the regulation of antigen receptor-mediated lipid raft dynamics. *Nat. Immunol.* **7:625-633**.

Neetu Gupta, and A. L. DeFranco. 2003. Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B cell activation. *Mol. Biol. Cell.* **14: 432-444**.

Neetu Gupta, A. M. Scharenberg, J-P. Kinet, and E. O. Long. 1998. The SH2-containing inositol phosphatase (SHIP) recruits the p85 subunit of phosphatidylinositol-3 kinase during Fc γ RIIb1-mediated inhibition of B cell receptor signaling. *J. Biol. Chem.* **274**: 7489-7494.

D. N. Burshtyn, A. S. Lam, M. Weston, **Neetu Gupta**, P. A. M. Warmerdam, E. O. Long. 1998. Conserved residues N-terminal of cytoplasmic tyrosines contribute to the Shp-1 mediated inhibitory function of killer cell Ig-like receptors. *J. Immunol.* **162**: 897-902.

Neetu Gupta, A. M. Scharenberg, D. N. Burshtyn, N. Wagtmann, M. N. Lioubin, L. R. Rohrschneider, J-P. Kinet, and E. O. Long. 1997. Negative signaling pathways of the killer cell inhibitory receptor and Fc γ RIIb1 require distinct phosphatases. *J. Exp. Med.* **186**:473-478.

Neetu Gupta, B. M. Martin, D. D. Metcalfe, and P. V. Subba Rao. 1996. Identification of a novel hydroxyproline-rich glycoprotein as the major allergen in *Parthenium* pollen. *J. Allergy Clin. Immunol.* **98**:903-912.

Neetu Gupta, P. Sriramaraao, R. Kori, and P. V. Subba Rao. 1995. Immunochemical characterization of rapid and slowly released allergens from the pollen of *Parthenium hysterophorus*. *Int. Arch. Allergy Immunol.* **107**:557-565.

Reviews

A. Viola and **Neetu Gupta**. 2007. Tether and Trap: Regulation of lipid raft dynamics by actin-binding proteins. *Nat. Rev. Immunol.* **7**:889-896.

Neetu Gupta, and A. L. DeFranco. 2007. Lipid rafts and B cell signaling. *Sem. Cell Dev. Biol.* **18**:616-626.

Book chapters

Neetu Gupta, A. L. DeFranco, and A. Weiss. Signal transduction by T and B lymphocyte antigen receptors. In Primary Immunodeficiency Diseases, a Molecular and Genetic Approach, 2nd Edition, H. D. Ochs, C. I. E. Smith, and J. Puck, eds., Oxford Press, Oxford, UK. **2007**.